

The University of Chicago

COCCUS FORMS OF CORYNEBACTERIUM DIPHTHERIAE

A PART OF A DISSERTATION SUB-
MITTED TO THE FACULTY OF THE
DIVISION OF THE BIOLOGICAL
SCIENCES IN CANDIDACY FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

1933

By
THOMAS CHRISTMAN GRUBB

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THOMAS C. GRUBB

CHICAGO

Coccus forms in cultures of the diphtheria bacillus have presented an interesting problem for many years. Although they were at first believed to be contaminants, careful technic proved that they could be found in pure cultures of the diphtheria organism. Later it was believed that these coccus forms represented involution or degenerate forms found in aging cultures or occurring when the organisms were subjected to unfavorable conditions.

The present tendency to regard atypical forms of many bacterial species as definite stages in a developmental cycle has led several workers to believe that the coccus forms of the diphtheria bacillus represent a stage in the life cycle of the organism. Therefore, the problem remains to determine whether these coccus forms represent transitory morphologic variations, resulting from unfavorable cultural conditions, or a stage in the life cycle of the organism. There is also the third possibility that these forms may be produced by both of the aforementioned processes, according to the condition of the culture.

In 1898 Slawyk and Manicatide¹ found three out of thirty recently isolated strains of the diphtheria bacillus which produced coccus forms on various mediums. Bernhardt² obtained atypical colonies containing coccus forms from cultures of *Corynebacterium diphtheriae* grown in normal human serum, guinea-pig serum or antitoxic horse serum. Heinemann³ observed the spontaneous appearance of coccus forms in a strain of Park 8. These cocci regained their bacillary form when transferred to Loeffler's medium. Mellon⁴ studied the coccus forms of a diphtheroid, *Corynebacterium Hodgkini*. He believed that these forms were involved in a life cycle of the organism. Kilian⁵ inoculated mice and guinea-pigs with diphtheria bacilli by injection and by feeding. When the organisms recovered from the abdominal cavity and lymph glands were cultured, many "degenerated" and coccus forms were found.

The most comprehensive study of coccus forms of *C. diphtheriae* was made by Yarisawa.⁶ Out of sixty-seven strains, 14 per cent produced coccus forms on blood agar. When these forms were transferred to serum agar or injected into guinea-pigs they resumed their original bacillary form. All the coccus-

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1. Ztschr. f. Hyg. u. Infektionskr. **29**: 181, 1898.

2. Ztschr. f. Hyg. u. Infektionskr. **79**: 179, 1915.

3. J. Bact. **2**: 361, 1917.

4. J. M. Research **49**: 9, 1920.

5. Ztschr. f. Hyg. u. Infektionskr. **102**: 262, 1924.

6. Japan M. World **6**: 35, 1926.

forming strains were toxigenic. Hadley⁷ stated that he had observed "distinctly coccoid types" when growing Park 8 on agar or in ascitic fluid.

Parish⁸ observed the formation of coccus forms of Park 8 on Hartley's tryptic digest medium. These forms reverted to bacilli when transferred to Loeffler's medium. Smith and Jordan⁹ aged nineteen strains of the diphtheria bacillus in broth for six months. When filtrates of these cultures were streaked on agar, there was an initial growth of coccus forms in eleven of the nineteen strains. Later there was a gradual transition of these cocci to the bacillary form. A year later these workers¹⁰ described the production of coccus forms with a bacteriophage-susceptible strain of *C. diphtheriae* when it was grown on Besredka's egg medium.

Kuschnarjew¹¹ grew diphtheria bacilli in nutrient broth containing varying quantities of magnesium, zinc, sodium, nickel, calcium and iron salts. In some cases, when these cultures were streaked on a meat-peptone-agar medium, rough colonies containing cocci were found. Kuhn and Sternberg¹² produced coccus forms of the diphtheria bacillus by subjecting it to various concentrations of "ammonia." When the organisms thus treated were streaked on agar, coccus forms were often observed. Some of these cocci were found to pass through Berkefeld N filters. Maver¹³ found various types of cocci when the diphtheria bacillus was grown on her synthetic medium. These cocci were antigenically different from the bacillary forms in the cultures from which they arose.

Pope and Pinfield¹⁴ described the production of coccus forms with a strain of Park 8 when it was grown on a tryptic digest of beef heart medium containing from 20 to 50 mg. of copper per liter. However, 100 per cent production of coccus forms was obtained only when the medium was sterilized by filtration. The cocci were poor toxin producers. Malcherek¹⁵ found that in mediums prepared from the livers of guinea-pigs weighing less than 200 gm., three diphtheria strains produced coccus forms, while three "pseudodiphtheria" strains remained in the bacillary form. Coccus forms were not produced when the livers of guinea-pigs weighing over 200 gm. were used. When the cocci were transferred to Loeffler's medium they regained their bacillary form. Malcherek suggested that this medium might be used to differentiate diphtheria from pseudodiphtheria strains.

Stone and Hobby¹⁶ observed the spontaneous alternation of the appearance of rods and cocci of several strains of the diphtheria bacillus when grown in a veal-proteose peptone medium. The organisms were very resistant to

7. J. Infect. Dis. **49**: 1, 1927.

8. Brit. J. Exper. Path. **8**: 162, 1927.

9. J. Bact. **20**: 25, 1930.

10. Smith, G. H., and Jordan, E. F.: J. Bact. **21**: 75, 1931.

11. Ztschr. f. Immunitätsforsch. u. exper. Therap. **68**: 210, 1930.

12. Zentralbl. f. Bakt. (Abt. 1) **121**: 113, 1931.

13. J. Infect. Dis. **49**: 9, 1931.

14. Brit. J. Exper. Path. **13**: 60, 1932.

15. Zentralbl. f. Bakt. (Abt. 1) **125**: 65, 1932.

16. J. Bact. **27**: 403, 1934.

diphtheria bacteriophage in the bacillus stage, but highly susceptible in the coccus stage. Apparently the coccus forms were more stable than the rod forms and showed little tendency to reversion on Loeffler's medium.

The following facts are evident from this review: The majority of the workers have not suggested that the coccus forms indicate the presence of a life cycle. There is a general disagreement concerning the toxigenesis of the coccus forms. With the exception of Stone and Hobby most workers have found that the coccus forms quickly revert to the bacillary forms on a favorable medium such as Loeffler's. Yarisawa was the only one to employ a sufficient number of strains to give statistical weight to his results. No one has studied the physical or chemical mechanism of the bacillus-to-coccus or coccus-to-bacillus change.

The experimental work to be described in this report is divided into two general sections. The first deals with the spontaneous appearance of coccus forms when an attempt was made to dissociate the diphtheria organism. The second describes the production of coccus forms at will in a liver infusion medium.

SPONTANEOUS APPEARANCE OF COCCUS FORMS

A series of experiments was planned to attempt the dissociation of the diphtheria bacillus into rough and smooth variants. While this work was in progress coccus forms were frequently observed. At that time their appearance was considered an interesting and somewhat mysterious change in cell morphology. Since it is desired only to point out the general conditions under which these coccus forms appeared spontaneously, the following experiments are not described in detail.

In the first series of experiments three freshly isolated strains, originally all in the bacillary form, were repeatedly transferred on 10 per cent sheep blood agar, on "chocolate" agar of the same composition, in 5 per cent casein digest broth and in veal infusion and liver infusion broths. The reaction of all the mediums was between p_H 7.2 and p_H 7.4. The repeated transfers on solid mediums were carried out by picking off colonies and streaking them on fresh plates at daily intervals. The repeated transfers in liquid mediums were made by transferring a loopful of the culture from tube to tube after a twenty-four hour incubation at 37 C. The colonial morphology of each transfer was checked daily by streaking a loopful of the contents of the tubes on blood or serum agar plates. The contents of the developing colonies were stained with Loeffler's methylene blue for microscopic study. The results of this series of experiments, so far as the appearance of coccus forms is concerned, can be summarized by stating that there was an irregular alternation between the appearance of bacillary and coccus forms in the colonies developing on the blood or serum agar plates. That is, for several days colonies containing only bacillary forms were found; during the next few days colonies containing either a mixture of bacilli and cocci or cocci alone were observed; then for the next few days only bacilli were seen in the developing colonies, etc. No correlation was found between the type of colony and the morphology of its constituent cells.

In the second series of experiments the aforementioned three strains were aged in casein digest, veal infusion and liver infusion broths. The flasks containing these cultures were capped with tin-foil and incubated at room temperature (from 22 to 25 C.). At various intervals covering a period of one month a loopful of the contents of the flasks was streaked on blood or serum agar. In this case again an irregular alternation between the appearance of bacillary and coccus forms was found in the developing colonies.

For the third series of experiments a quantity of unconcentrated diphtheria antitoxin containing 600 units per cubic centimeter and a similar quantity of normal horse serum were ob-

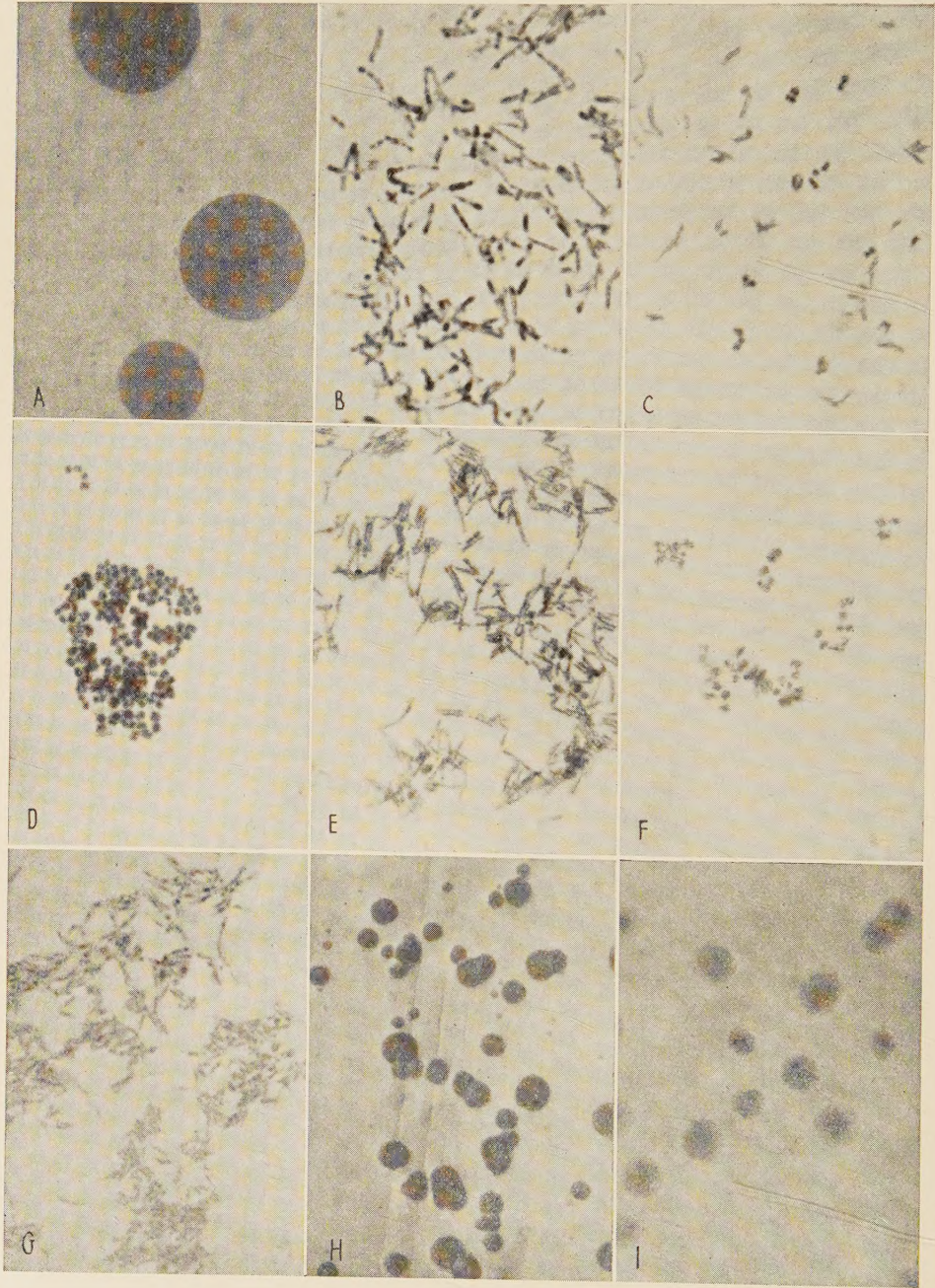
tained. Two series of three flasks, each containing 100 cc. of veal infusion broth (p_H 7.2), were prepared. Ten per cent antitoxin was added to the first flask and 10 per cent normal horse serum to the second. The third flask served as a control. A smooth strain of Park 8 was inoculated into one series and a recently isolated smooth strain into the other. The flasks were capped with tin-foil and incubated at 37 C. for two weeks and then at room temperature for four months. At daily intervals during the first two weeks and at weekly intervals thereafter the contents of the flasks were studied by a direct microscopic examination and by streaking on serum agar plates. The microscopic examination of the contents of all the flasks and the contents of the colonies developing on the serum agar plates again showed an irregular alternating occurrence of bacillary and coccus forms. The presence of the antitoxin or of the normal horse serum did not account for the production of the coccus forms since they appeared in the control flasks with equal frequency.

In the fourth series of experiments a smooth strain of Park 8 was aged two weeks at 37 C. in veal infusion broth containing 30 per cent antibacterial (S) serum. The antiserum was prepared by injecting a smooth strain of Park 8 into a rabbit until an agglutination titer of 1:8,000 was obtained. A second series of flasks was prepared in which a smooth, recently isolated strain was aged for two weeks in veal infusion broth containing 30 per cent antibacterial (R) serum. The R antiserum was obtained by injecting into a rabbit a previously dissociated rough strain until an agglutination titer of 1:4,000 was reached. As controls, flasks containing similar quantities of normal rabbit serum (from the animals before immunization) and broth alone were included in the series. The contents of the flasks were examined in the manner described in the previous experiment. Again an irregular alternation between the appearance of the bacillary and coccus forms was observed both in the contents of the flask and in the colonies developing on the serum agar plates. The presence of antiserum or normal serum did not account for the production of coccus forms since they were also found in the control flasks.

An interesting occurrence in these series of experiments was the appearance of small-colony variants or G colonies similar to those described by Hadley and his co-workers.¹⁷ These colonies appeared on the serum agar plates streaked from broth containing the anti-R serum, normal rabbit serum and broth control. They appeared on the plates streaked from the liquid cultures from the fifth to the tenth day of aging and did not reappear throughout the remainder of the aging period. Figure 1A shows the relative size of the G and the normal S colony. The cellular content of the smaller colony consisted of short, solidly stained rods while that of the larger colony consisted of about equal numbers of rods and cocci. When the small colony was picked off and streaked on a second serum agar plate a few of the developing colonies remained small, while the majority of them grew to the normal size. When a small colony was picked from this plate and streaked on a third plate, a still larger percentage of normal S colonies appeared. When this procedure was repeated on the fourth plate, only normal S colonies developed. An attempt was made to preserve the small colonies by transferring them to blood agar slants which were stored in the icebox. However, when these cultures were streaked out several weeks later, only normal S colonies appeared.

Briefly summarizing this section, it is to be noted first that coccus forms of the diphtheria bacillus may appear apparently spontaneously under a variety of cultural conditions. Daily observations on aging or repeated transfer cultures disclosed an irregular alternation of bacillary and coccus forms. Figures 1B, C and D illustrate the appearance of the bacillary and coccus forms as they occurred in some of the repeated transfer series. The coccus forms are unlike staphylococci since they occur characteristically as diplococci or tetrads. A tempting explanation of the alternation of the bacillary and coccus forms is that a life cycle is involved in which these forms represent successive cyclic stages. In the light of the experimental work to be described in the second section of this report, a more plausible explanation for this

17. J. Infect. Dis. 48: 1, 1931.



alternation in morphology will be suggested. Finally it should be added that although the primary purpose of the foregoing work was to dissociate the diphtheria organism, only in a few instances were rough colonies observed. This observation is contrary to the writings of many authors who imply that microbic dissociation is a universal and easily induced phenomenon.

PRODUCTION OF COCCUS FORMS IN LIVER INFUSION MEDIUMS

It was observed that when a culture of *C. diphtheriae* containing only bacillary forms was transferred to liver infusion agar, the bacillary forms were completely replaced by almost a pure culture of coccus forms after twenty-four hours at 37 C. Since this rapid and facile morphologic transformation was quite unusual, it was decided to investigate this change more thoroughly. A careful study showed that this morphologic change was not a chance observation but could be duplicated with several strains. Thus an opportunity presented itself to study the virulence, toxigenesis, filtrability and fermentation reactions of the coccus forms of the diphtheria bacillus.

Liver Infusion Medium.—The details of preparing the liver infusion medium are as follows:

Five hundred grams of fresh, chopped beef liver were infused overnight in 1 liter of distilled water. This infusion was filtered through cheesecloth and the filtrate boiled five minutes. The coagulated proteins were removed by a second filtration through cheesecloth and 1 per cent peptone and 0.5 per cent sodium chloride were added to the filtrate which was heated gently to bring these ingredients into solution. If liver infusion agar was to be prepared, 2 per cent agar was added at this point. After all the constituents had been brought into solution, the medium was cooled to from 40 to 45 C. and 1 per cent powdered egg albumin was added for clarification. Sufficient distilled water was then added to bring the final volume to 1 liter, and the medium was filtered through glass wool. The reaction was adjusted to p_H 7.4, and the medium was autoclaved at 15 pounds (6.8 kg.) for fifteen minutes, bringing the final reaction to about p_H 7.2.

Morphologic Changes with Several Strains on Various Mediums.—After the initial discovery that coccus forms were produced on liver infusion agar it was necessary to determine how many other *C. diphtheriae* strains would

Fig. 1.—Unless otherwise stated, all the specimens were stained with Loeffler's methylene blue and photographed at a magnification of 1,425 diameters. *A*, numerous small colony variants and several normal-sized colonies on a serum agar plate streaked from a flask containing veal infusion broth ($\times 30$). *B*, bacilli found in the first eighteen transfers of a recently isolated strain on chocolate agar. *C*, bacilli, diplococci and tetrads found in the nineteenth to twenty-second transfer of the foregoing strain on chocolate agar. *D*, 100 per cent coccus forms found in some of the colonies developing on serum agar which had been streaked from the repeated transfer cultures in veal infusion broth. *E*, twenty-four hour growth of strain 2 on blood agar showing the predominance of bacillary forms. *F*, twenty-four hour growth of strain 2 on liver infusion agar after being transferred from the aforementioned blood agar culture. Note the characteristic large and small diplococci. *G*, twenty-four hour growth of strain 2 on serum agar after being transferred from the aforementioned liver infusion agar culture. Note the predominance of bacillary forms with a few remaining coccus forms. *H*, thirty-six hour growth of strain II on serum agar. The colonies are smooth, round and raised ($\times 30$). *I*, thirty-six hour growth of strain II on liver infusion agar. The colonies are rough, flat, irregular, small and have a central thickening ($\times 60$). Note the difference in magnification.

show this change and also what effect other mediums would have on the coccus forms.

Eleven recently isolated strains and a stock strain of Park 8, all in the bacillary form on blood agar slants, were transferred to liver infusion agar slants and incubated at 37 C. for twenty-four hours. At the end of this period the growth on all but one of the slants contained only coccus forms. Each of these strains was then transferred to serum agar slants and after twenty-four hours' incubation, all the tubes contained only bacillary forms. Figure 1E shows one of these strains in the bacillary form on a blood agar slant. When this culture was transferred to liver infusion agar only coccus forms were found after twenty-four hours' incubation, as shown in figure 1F. Figure 1G shows how these coccus forms reverted to the bacillary form after twenty-four hours' growth on serum agar. This morphologic transformation was typical for eleven of the twelve strains studied and could be repeated at will. Regarding the one strain which did not change to the coccus form on liver infusion agar, it will be shown later that certain strains do not exhibit this morphologic change; apparently one of these strains was included in this series.

The growth of all the strains on liver infusion agar is extremely scanty, being comparable to the growth of a "fastidious" streptococcus on nutrient agar. The comparative size and morphologic characteristics of the colonies produced on serum agar and liver infusion agar are shown in figures 1H and I.

When coccus forms on liver infusion agar were transferred to veal infusion broth, veal infusion agar, nutrient broth, nutrient agar and Loeffler's medium they reverted to the bacillary form after suitable incubation.

Although, as is well known, the bacillary forms of every strain of the diphtheria bacillus exhibit a characteristic morphology (i.e., Wesbrook types), the cocci produced in liver mediums are morphologically similar for all the strains. They occur as large and small diplococci and tetrads. When cocci are produced in liver mediums the conversion is usually complete, although a few strains were found which showed only from 50 to 75 per cent conversion to the coccus form. While the coccus forms developing in liver infusion broth show a tendency to revert to the bacillary form after a few days, the coccus forms produced on liver infusion agar have remained in that form as long as they have been under observation, a period of from three to four weeks. Coccus forms may be repeatedly transferred on liver infusion agar without reverting to the bacillary form; but after any number of such transfers they revert to the bacillary form when transferred to a favorable medium. Dehydrated liver infusion medium has not been found to promote the production of coccus forms.

Direct Observation of the Development of Coccus Forms.—While several workers have suggested that the coccus forms of the diphtheria bacillus were merely very short bacilli, no one has determined the physical mechanism of their development. Since the production of coccus forms on liver infusion agar is so certain and rapid, an opportunity for determining the mechanism of their production offered itself. For this purpose a hanging block of liver infusion agar on a cover slip was inoculated with bacillary forms of the organism and inverted in a well slide on a warm stage. Microscopic observations and photographs were made on this preparation at various intervals over a period of twenty-six hours. During the first seven hours the bacilli divided by the usual binary fission. Toward the end of the eighth hour most of the

cells began to contract into short, plump rods which finally became rounded, coccoid forms. For the remainder of the period of observation the cocci divided by binary fission. Figures 2D to G show the various stages of development of the coccus forms on the hanging block. These photographs do not show the complete conversion of all the bacilli into cocci as it occurs in a liver infusion agar slant probably because of lack of sufficient water and nutrient material in the thin layer of medium. It is also obvious that a number of dead cells which show no change have been included in the field.

In order to show the progressive changes that occur when the bacillary form is grown on liver infusion agar slants at 37 C. samples of growth were removed at various periods of incubation. Figures 2, A to C show three definite stages in the conversion. By comparing figures 2D to G with figures 2A to C it is seen that the conversion is much more rapid and complete on the agar slant than on the hanging block.

To study the coccus-to-bacillus change a similar direct microscopic observation was made on a hanging block of veal infusion agar which had been inoculated with coccus forms. After a few hours the cocci elongated into rods and then divided by the usual binary fission.

The direct microscopic observations made it clear that the morphologic changes were purely physical transformations, and no suggestion of a life cycle was observed.

Virulence of Coccus Forms.—Determination of the virulence of coccus forms would appear to be simply a matter of inoculating experimental animals. Several experiments of this type were done, and it was found that if the bacillary form of the strain was virulent, the coccus form was also virulent. Conversely, strains which were avirulent in their bacillary form were also avirulent in their corresponding coccus form, as demonstrated by intradermal injections into guinea-pigs. However, when some of the material from the intradermal lesions was aspirated it was found that the injected coccus forms changed into the bacillary forms within at least eight hours after the injection. Even when melted liver infusion agar was injected subcutaneously into a guinea-pig and the coccus forms were inoculated into this agar focus, the cocci reverted to bacilli in a short time. Therefore one can draw no valid conclusions concerning the virulence of coccus forms from these experiments. It is certain, nevertheless, that passage of a given strain through the coccus stage does not alter its virulence potentialities one way or the other.

Toxigenesis of Coccus Forms.—Six strains of *C. diphtheriae* were grown in flasks containing veal infusion and liver broth. After seven days' incubation at 37 C. the filtrates were inoculated intradermally into guinea-pigs to test for the presence of toxin. The strains which were toxigenic in the veal infusion broth were nontoxigenic in the liver infusion broth. However, it was found that the initial growth of coccus forms in the liver infusion broth gave way to the presence of only bacillary forms after three or four days' incubation. Thus the toxigenesis of coccus forms was not being tested, but in reality that of bacilli which had passed through the coccus stage.

It was also observed that the liver infusion broth cultures became distinctly acid and remained so during the period of incubation, while the veal



infusion broth cultures showed a transient acidity and then became alkaline. Since it is known that an acid reaction inhibits the production of toxin, it was believed that this observation accounted for the lack of toxin production on the part of the known toxigenic strains in liver infusion broth. The high acidity of the broth can be easily accounted for by the probable fermentation of the hydrolytic products of glycogen in the broth.

The fermentation reactions of the coccus forms in liver infusion broth could not be determined because the broth itself contained sufficient fermentable sugars to mask the action of the organism on the particular sugar that was added.

Filtrability of Coccus Forms.—Eight strains were grown on liver infusion agar and on Loeffler's slants for twenty-four hours at 37 C. Stained preparations showed that all the organisms on the Loeffler's slants were in the bacillary form, while all those on the liver infusion agar slants were in the coccus form. The growth on each slant was washed off with sterile veal infusion broth. Three cubic centimeters of this suspension were diluted with 10 cc. of sterile veal infusion broth and mixed well with 2 cc. of a twenty-four hour broth culture of *Chromobacterium prodigiosum* and 4 cc. of sterile ox serum. This suspension was then passed through Berkefeld N, W and Seitz filters. The ox serum and broth were added to the suspension to provide a favorable medium in the filtrate for the growth of any filter-passers. *Ch. prodigiosum* was used as the test organism to detect mechanical flaws in the filters. The filtrates were collected in sterile tubes placed inside the suction flasks, and after filtration the tubes were removed and plugged with sterile cotton.

All the filtrates were tested by the Hauduroy¹⁸ technic for eight successive transfers. The remainder of each filtrate in the original tube was capped with tin-foil and incubated at 37 C. for four weeks. Any turbidity developing in these tubes during this period was examined to identify the nature of the growth. At the end of the four weeks a loopful of the contents of all the tubes in which turbidity did not develop was transferred to Loeffler's slants, incubated twenty-four hours, then transferred to a second series of Loeffler's slants and again incubated twenty-four hours. In further experiments the Hauduroy technic was omitted and only Berkefeld N filters were employed.

Since the results of all the experiments were essentially negative, they

Fig. 2.—Unless otherwise stated, all the specimens were stained with Loeffler's methylene blue and photographed at a magnification of 1,425 diameters. *A*, strain 6 after two hours' incubation on a liver infusion agar slant. *B*, strain 6 after four hours' incubation on a liver infusion agar slant. Note several remaining bacillary forms. *C*, strain 6 after ten hours' incubation on a liver infusion agar slant. The bacillary forms have been completely replaced by coccus forms. *D* to *G*, unstained organisms in a hanging block of liver infusion agar on a warm stage ($\times 1425$). The time intervals of incubation at which these photographs were taken are as follows: *D*, two hours; *E*, seven hours; *F*, fourteen hours; *G*, twenty-six hours. *H*, twenty-four hour growth of strain 12 on Loeffler's medium. *I*, twenty-four hour growth of strain 12 on veal infusion agar containing 2 per cent ox bile. Note the swollen intracellular granules. *J*, twenty-four hour growth of strain 12 on veal infusion agar containing 4 per cent ox bile. The granules have swollen to such an extent that they simulate the appearance of cocci.

are briefly summarized. Although growth was observed on several of the Hauduroy plates, it was obvious that it represented only air contamination since the isolated organisms were chiefly gram-positive, sporulating bacilli. Wyckoff¹⁹ recently made the apt remark that this technic "courts" contamination. Turbidity developed in several of the filtrates after twenty-four hours' incubation, but in all the cases only the test organism was isolated, probably indicating a leaky filter. Results obtained with these filters were disregarded. In five of the filtrates turbidity developed after two weeks' incubation. No organisms could be seen in these filtrates, and when they were transferred three successive times on Loeffler's slants no growth was obtained. The cause of this turbidity is not known, but it probably represents precipitation of some of the proteins in the filtrate. The majority of the filtrates remained clear for the entire period of incubation.

In conclusion it may be stated that no evidence was found that the diphtheria bacillus, in either the bacillary or the coccus form, was filtrable under the conditions of this experiment.

Differentiation of Diphtheria and Diphtheroid Organisms on Liver Infusion Agar.—In a preliminary experiment seventeen strains reported to be diphtheria organisms and eleven strains reported to be diphtheroid organisms were inoculated on liver infusion agar slants and incubated for twenty-four hours at 37 C. At the end of that period all the diphtheria strains had reverted to the coccus form, while all the diphtheroid strains remained in the bacillary form. Since this observation suggested a possibility of a simple cultural test for distinguishing between these two groups of organisms, it was decided to make a more complete investigation of this test.

Ninety-six strains of diphtheria and diphtheroid organisms were collected from various parts of the country. The morphology as well as the virulence and fermentation reactions of each strain were studied with great care. Thirty-one strains were found to be virulent diphtheria strains, while twenty-six were classed as avirulent diphtheria strains and thirty-nine as diphtheroid strains. All the strains were grown on liver infusion agar slants for twenty-four hours at 37 C. The number producing coccus forms is indicated in the table.

Coccus Forms Produced by Diphtheria and Diphtheroid Strains on Liver Infusion Agar

Number of Strains	Number of Strains Producing Cocci	Percentage
31 virulent diphtheria	13	43
26 avirulent diphtheria	10	39
39 diphtheroid	2	

Since only 40 per cent of the diphtheria strains and 5 per cent of the diphtheroid strains produced coccus forms on liver infusion agar it is obvious that this test cannot be used as a practical diagnostic method.

The most striking observation in this experiment was that the diphtheria strains which produced coccus forms showed a marked uniformity in their fermentation reactions. They attacked dextrose, maltose and dextrin and failed to ferment lactose, sucrose and mannitol. The diphtheria strains which did not produce cocci did not fall into a definite fermentation group but va-

19. J. Exper. Med. 57: 165, 1933.

ried in their reactions on the different sugars. It is believed that these results indicate a distinct physiologic difference between the coccus and the non-coccus-forming diphtheria strains.

While this work was in progress, Malcherek¹⁵ reported that three strains of diphtheria bacilli produced coccus forms on guinea-pig liver infusion agar while three strains of "pseudo-diphtheria" bacilli did not show this change. She suggested that this medium might be used to differentiate the diphtheria from the pseudodiphtheria strains.

The results of the present study indicate that Malcherek was apparently working with three diphtheria strains which produced cocci on liver infusion agar. It seems probable that if she had used a larger number of strains the correlation which she obtained would not have been so striking.

COCCUS FORMS PRODUCED BY THE METHODS OF OTHER WORKERS

In order to determine how the coccus forms on liver infusion agar compared with those described by other workers an attempt was made to produce coccus forms by the methods of these workers.

Following the methods of Yarisawa,⁸ eleven strains of diphtheria bacilli which produced coccus forms on liver infusion agar were streaked on rabbit, sheep and guinea-pig blood agar plates. Two of these strains produced cocci on the guinea-pig blood agar, thus confirming the results of Yarisawa to a limited extent.

Ten diphtheria strains were inoculated into a veal infusion broth containing 0.2 per cent dextrose and 1.5 per cent agar, which Heinemann³ found to produce coccus forms. Two of the strains showed a partial conversion to coccus forms in this medium.

Mellon⁴ observed that coccus forms of *C. Hodgkini* were formed in nutrient broth containing 1 per cent maltose at p_H 6.6. Ten strains were inoculated into this medium with the result that one of them produced a pure culture of coccus forms.

In Hadley's monograph on dissociation, he mentions observing the formation of cocci with a strain of Park 8 in a medium containing ascitic fluid. Although ten strains were inoculated into a veal infusion broth containing 50 per cent of fresh ascitic fluid, coccus forms were not found in any of the cultures after suitable incubation.

Pope and Pinfield¹⁴ observed that when from 10 to 50 mg. of copper (in the form of copper sulphate) were added to 1 liter of tryptic digest of beef heart medium sterilized by filtration, a pure culture of coccus forms of Park 8 was produced. Similar amounts of copper, sterilized separately by autoclaving, were added to veal infusion broth and to a tryptic digest of beef heart medium. Two strains were inoculated into these mediums, and after twenty-four hours' incubation at 37 C. a mixture of coccus and bacillary forms was seen in the cultures containing from 10 to 40 mg. of copper. Growth was completely inhibited in the cultures containing 50 mg. of copper.

In all the cases in which coccus forms were produced by any of the foregoing methods they were morphologically indistinguishable from those produced on liver infusion agar. However, none of these mediums promoted coccus formation to as marked a degree as, or with the constancy exhibited by, the liver infusion mediums.

INGREDIENTS OF LIVER INFUSION MEDIUMS PRODUCING COCCUS FORMS

A preliminary study was made to determine what factor or factors in liver infusion mediums promoted coccus formation.

Various concentrations of glycogen, dextrose and bile were added to veal infusion broth and several coccus-forming strains inoculated. The results were generally unsuccessful with the exception of one instance in which bile caused the intracellular granules to swell to such an extent that they simulated cocci (figs. 2I and J). Several attempts were made to repeat this experiment, but only bacilli with slightly enlarged granules were observed.

COMMENT

Early in this report it was suggested that the spontaneous appearance of coccus forms in aging and repeatedly transferred cultures might be considered as evidence for the presence of a life cycle of the diphtheria bacillus. However, in the light of the results obtained with the liver infusion mediums it is believed that the following reasoning offers a more plausible explanation for the spontaneous appearance of coccus forms than the postulation of the presence of a life cycle.

The fact that the diphtheria bacillus can be readily transformed into a coccus form in liver infusion mediums, and with equal ease changed back into a rod on favorable mediums, conclusively indicates that a physical or chemical difference in the menstruum may be all that is necessary to produce these morphologic changes. From the early experiments with aging and repeated transfer cultures it is evident that the physical and chemical composition of these cultures is never static, owing to the accumulation of growth products, dehydration, change in reaction, etc. Therefore it is logical to conclude that these intrinsic variations in the medium may be sufficient to cause the production of coccus forms, with the subsequent transformation to bacillary forms as the composition of the medium changes. This would account for the apparently spontaneous alternation of morphologic types.

Since it has been shown that there are certain strains which do not exhibit any morphologic change in liver infusion mediums, it is evident that there are some strains which apparently are indifferent to changes in the composition of the medium, thus accounting for the fact that coccus forms are not found in every culture of the diphtheria bacillus.

When the photomicrographs of the coccus forms found in the repeated transfer cultures are compared with those showing the coccus forms developing on liver infusion agar, no striking morphologic differences are observed. This fact suggests that the mechanism of their production is similar.

In conclusion it may be stated that throughout this work no evidence for postulating a life cycle to explain coccus formation was found. All observations point to the conclusion that coccus forms are produced by a simple, physical change under the stimulus of some undetermined factor or factors in certain mediums. These results are in accordance with the conclusions of a growing number of workers who are finding that morphologic variation may indicate nothing more than unfavorable cultural conditions.²⁰

While these results do not preclude the possibility that some coccus forms

20. Rettger, L. F., and Gillespie, H. B.: *J. Bact.* **26**: 289, 1933. Lewis, I. M.: *Ibid.* **24**: 381, 1932; **25**: 359, 1933. Hussong, R. V.: *ibid.* **25**: 537, 1933. Wyckoff, R. W. G.: *J. Exper. Med.* **57**: 176, 1933. Wyckoff, R. W. G., and Smithburn, K. C.: *J. Infect. Dis.* **53**: 201, 1933. Knaysi, G.: *J. Bact.* **26**: 623, 1933.

may play a rôle in the yet unproved life cycle of *C. diphtheriae*, they offer an explanation for the production of coccus forms which is in accord with the more firmly established bacteriologic principle that transitory morphologic variations may be produced by certain changes in the cultural conditions.

SUMMARY

An irregular alternation between the appearance of bacillary and coccus forms of the diphtheria bacillus was found when it was aged or repeatedly transferred in various mediums.

A small colony variant or "G" colony was observed which did not appear to be related to a life cycle or to the production of coccus forms.

Coccus forms of certain strains were produced at will by growing them in liver infusion mediums. These cocci reverted to bacilli when transferred to favorable mediums.

A direct microscopic observation on the warm stage was made of the bacillus-to-coccus and coccus-to-bacillus change.

The virulence, toxigenesis and fermentation reactions of the coccus forms could not be satisfactorily determined.

The differentiation of diphtheria and diphtheroid strains on liver infusion agar was not cleancut enough for diagnostic purposes.

When methods employed by other workers for producing coccus forms were successfully duplicated, the resulting cocci were morphologically indistinguishable from those produced in liver infusion mediums.

The addition of glycogen, dextrose or bile to veal infusion broth did not induce the formation of cocci.

